hexenal, 6728-26-3; *cis*-3-hexen-1-ol, 928-96-1; 1-hexanol, 111-27-3; *trans*-2-hexen-1-ol, 928-95-0; 2-heptanol, 543-49-7; 3-butenyl isothiocyanate, 3386-97-8; 2,3-octanedione, 585-25-1; myrcene, 123-35-3; *cis*-3-hexenyl acetate, 3681-71-8; 1-hexyl acetate, 142-92-7; *trans*-2-hexenyl acetate, 2497-18-9; *p*-cymene, 99-87-6; limonene, 138-86-3; ocimene, 29714-87-2; *cis*-3-hexenyl propionate, 33467-74-2; nonanal, 124-19-6; linalool, 78-70-6; *cis*-3-hexenyl butyrate, 16491-36-4.

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Fate of Avermectin B₁a in Soil and Plants

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Laboratory studies of the fate of ^{3}H-labeled avermectin B₁a (AVM) in Lufkin fine sandy loam, Houston clay, and coarse sand demonstrated that under aerobic conditions the compound was degraded at a fairly rapid rate (respective half-lives of AVM in the three soils at a 1-ppm treatment rate were ca. 14–28, 28–56, and 56 days) to at least 13 radioactive products. The major soil degradation product was an equilibrium mixture (ratio of 1:2.5) of the 8α-hydroxy derivative and the corresponding ring-opened aldehyde derivative of AVM. AVM did not leach in any of the three soil types. In cotton seedlings grown either in aged or unaged samples of Lufkin fine sandy loam treated with [^{3}H]AVM (10 ppm) little (≤0.1 ppm) radioactive material was absorbed by roots and translocated to the aerial portions of the plant. Surface residues of [^{3}H]AVM applied to individual cotton leaves in the field were rapidly depleted (half-life < 1 day) and little radioactive material (maximum of ca. 8%) was absorbed by treated leaves. Field tests of a fire ant bait formulation of [^{14}C]AVM applied to the soil furface at rates of 50, 100, and 500 mg of AI/acre indicated that little if any radioactive material (<0.01 ppm) was taken up by Bermuda grass grown in the treated areas.

The insecticide/acaricide avermectin B₁a (AVM, Figure 1) is one of several macrocyclic lactones formed during microbial fermentation reactions involving the actinomycete *Streptomyces avermitilis* (Burg et al., 1979). AVM is a neurotoxin that is thought to manifest its action by disrupting the normal function of γ-aminobutyric acid (GABA), an important neurotransmitter in the central nervous system of vertebrates and in the peripheral nervous system of invertebrates. In vitro studies with preparations of rat brain have shown that AVM stimulates presynaptic release of GABA (Pong et al., 1980) and enhances postsynaptic binding of GABA (Pong and Wang, 1982); the action of AVM is antagonized by bicuculline and picrotoxin. AVM is quite toxic to mammals; the acute oral LD₂₀ (mice and rats) is 10–30 mg/kg and the acute dermal LD₅₀ (rats and rabbits) is <400 mg/kg. However, the exceptionally high toxicity of this compound to certain arthropods is such that use patterns can probably be developed so that acute hazards to mammals and other nontarget organisms will be greatly minimized (Putter et al., 1981).

Cotton Insects Research Laboratory (D.L.B.) and Veterinary Toxicology and Entomology Research Laboratory (G.W.I.), Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas 77841, and Merck & Co., Inc., Three Bridges, New Jersey 08887 (J.G.M., V.F.G., and C.C.K.), and Rahway, New Jersey 07065 (B.H.A., J.M.S., and W.J.H.).

AVM is being investigated for possible use in controlling different phytophagous pests of field crops and citrus (Ku, 1983) and fire ants (Lofgren and Williams, 1982). Our report contains the results of studies of the fate of AVM after application to laboratory and field soils and to cotton plants. Specific tests reported on here include (1) laboratory studies with three types of soil to characterize the leaching of radiolabeled AVM and its degradation over time following application at different concentrations, (2) greenhouse studies to determine if [^{3}H]AVM or its radioactive degradation products are taken up by cotton seedlings grown in treated soil, (3) field studies of the absorption and degradation of [^{3}H]AVM after foliar application to cotton, and (4) field studies to determine the fate of [^{14}C]AVM after its application in a fire ant bait formulation to the soil surface and to evaluate the possible uptake of AVM or its radioactive degradation products by grass grown in treated areas.

MATERIALS AND METHODS

Chemicals. Samples of technical (>95% pure) AVM, unlabeled or radiolabeled with ^{3}H at the 5-position (specific activity 1.74 mCi/mg) or with ^{14}C at the 3-, 7-, 11-, 13-, and 23-positions (specific activity 16.4 μCi/mg), were provided by Merck & Co., Inc., Rahway, NJ. Also provided was a bait formulation (vide infra) of [^{14}C]AVM comparable to one that is being tested for efficacy against fire ants. Unlabeled samples of two potential degradation products, the 5-ketone and monosaccharide derivatives of AVM, were made available for use as an analytical standards.
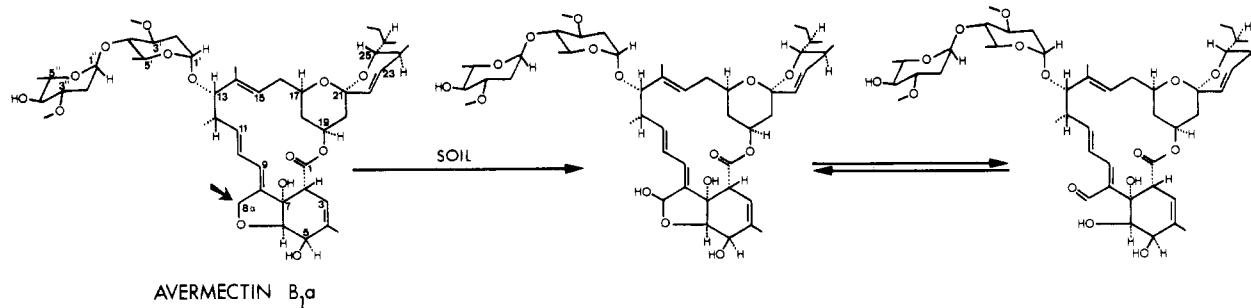
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Figure 1. Structures of avermectin B₁a and two of its degradation products in soil.

Soils and Plants. The three soil types used for laboratory studies were Lufkin fine sandy loam, Houston clay, and a construction-grade sand. Properties of these soils have been reported previously by Bull and Ivie (1982). Prior to use, the soils were air-dried and sieved to pass a 35-mesh screen.

Cotton plants used for different tests of the fate of [³H]AVM were of the Stoneville 213 variety grown either in the field with standard production procedures or in the greenhouse.

Two small plots (130 × 335 cm/each) of common Bermuda grass grown in Lufkin fine sandy loam were used for the field tests with a fire ant bait formulation of [¹⁴C]AVM. The individual plots were surrounded by wood boards (lined with plastic film to minimize possible runoff of radioactive material) that were buried in the ground to a depth of 20 cm. Plots were positioned 130 cm apart, and each plot was partitioned with plastic-lined boards as above to form three subplots of similar size.

Solutions and Formulations. Acetone solutions of radiolabeled AVM were used for soil treatments. The [³H]AVM used for treatments at the 1-, 10-, and 50-ppm levels was diluted ca. 10-fold with unlabeled AVM, while that used for treatments at the 0.1-ppm level was undiluted. [³H]AVM used for treatments of cotton leaves was diluted 5-fold with the unlabeled chemical and made up in a 2:1 mixture of ethanol and water.

The fire ant bait was a standard pregel defatted corn grit formulation consisting of 0.011% [¹⁴C]AVM, 30% soybean oil, and 69.989% pregel defatted corn grit granules. On the basis of the average weight of ca. 2.6 mg/granule, each granule contained ca. 0.29 μg of [¹⁴C]AVM.

Treatments. *Laboratory Soil.* Sufficient numbers of samples of each soil were weighed (10 g/sample) into standard glass scintillation vials. Each sample was treated with 200 μL of the appropriate solution of [³H]AVM to give a dry weight concentration in soil of 0.1, 1.0, or 50 ppm. After the solvent evaporated (usually within 30 min), vials were capped and the contents mixed thoroughly. Vials were then uncapped and sufficient distilled water was added to each sample to give a moisture content of ca. 75% of the 0.33-bar retention level (field capacity). These vials were recapped and allowed to stand 24 h to allow the moisture to distribute uniformly throughout the soil ("0-h" samples were processed immediately). Vials were then uncapped and held at ambient temperature and ca. 90% relative humidity (RH) in vented glass containers in a fume hood.

At the onset of this investigation, a short-term study of soil treated with [³H]AVM was conducted to evaluate the possibility of tritium exchange under the conditions of these studies. Triplicate samples of Lufkin fine sandy loam (10 g/each), treated as described with [³H]AVM at a rate of 10 ppm, were extracted at 0 and 28 days posttreatment. Extracts were stored at -70 °C until all were collected, and then each sample was analyzed by two-dimensional

TLC/autofluorography and HPLC.

Studies were made of the fate of [¹⁴C]AVM in Lufkin fine sandy loam under aerobic and anaerobic conditions. Samples (10 g) were treated as described above at a rate of 1.0 ppm. One group of these samples was set up for evaluations of the fate of [¹⁴C]AVM under the aerobic conditions described. In another set of samples, anaerobic conditions were established immediately after treatment; containers were flushed with nitrogen, and then the soil was flooded with distilled water and the vials were sealed until they were opened for analyses at definite times posttreatment. Other treated samples were held under aerobic conditions for 1 month and then anaerobic conditions were established as described and maintained for 3 months. These samples were processed and analyzed at definite times posttreatment as described.

For use in additional studies, a large sample (500 g) of Lufkin fine sandy loam was treated with 5 mL of an acetone solution of [³H]AVM to produce a dry weight concentration of 10 ppm of AVM in the soil. This solution was applied to the surface of the soil that was contained in a 1-L beaker. After the solvent evaporated, the treated soil was mixed thoroughly and then sufficient water was added to adjust the moisture content to field capacity. The treated sample was covered with aluminum foil, which had a few small holes (ca. 1-2 mm) to allow some ventilation, and stored in a fume hood at ambient temperature. This sample was weighed periodically to determine moisture loss and water was added as needed to maintain initial levels.

Leaching. The leaching of [³H]AVM in Lufkin fine sandy loam, Houston clay, and sand was determined with a soil TLC technique similar to that of Helling (1971). For this, 20 × 20 cm glass plates were coated with soil (500–750-μm layer) by using a standard gel spreader. After the soil had air-dried, [³H]AVM (2 μg in 5 μL of acetone) was applied to each soil plate. Radiocarbon-labeled samples of potassium 3,4-dichloro-5-isothiazolecarboxylate (PDIC) and diflubenzuron [N-[[(4-chlorophenyl)amino]-carbonyl]-2,6-difluorobenzamide] were also included in each analysis as standards of reference. (Respective water solubilities of these two chemicals are, for PDIC, 485 mg/mL and, for diflubenzuron, 0.0002 mg/mL.) Treated plates were developed in water until the solvent fronts migrated 10 cm from the point of sample application. The plates were then dried and treated with PPO (vide infra) and exposed to X-ray films for autofluorographic characterization of leaching patterns. Tests were replicated 3 times.

Biometer Flask Study. The biometer flask procedure of Bartha and Pramer (1965), as modified by Mansager et al. (1979), was used to determine if [¹⁴C]AVM was degraded in soil to ¹⁴CO₂. Four samples of Lufkin fine sandy loam (50 g) were each mixed with 500 μg of [¹⁴C]AVM and then placed in glass biometer flasks (Bellco Glass, Inc., Vineland NJ) and moistened with 5 mL of distilled water. Fifteen milliliters of 1 N sodium hydroxide was added to

the side arm of each flask, and then flasks were sealed and held at room temperature (ca. 25 °C) in an area where they were protected from exposure to light. At weekly intervals posttreatment, the trapping solution was removed for radiocarbon analysis and replaced with fresh trapping solution.

Radiocarbon in the trapping solutions of biometer flasks was radioassayed directly by LSC. The presence of $^{14}CO_2$ was verified by adding a saturated solution of barium chloride and radioassaying precipitated barium [^{14}C]-carbonate as described by Mansager et al. (1979). Also, some of the trapping solution was acidified with 6 N HCl and radioassayed again. In this case, a reduction in radiocarbon content of the solution would indicate that $^{14}CO_2$ was released as a result of the reaction of the acid with [^{14}C]sodium carbonate. This acidified solution was also extracted with methylene chloride and analyzed as described to determine the possible presence of organosoluble radioactive products.

Field Soil. Duplicate subplots of common Bermuda grass were treated in Sept 1982 with the pregel defatted corn grit bait formulation of [^{14}C]AVM at rates equivalent to 50, 150, and 500 mg of AI/acre (0.405 ha). For this, the number of granules needed per unit area was calculated for each treatment level and then the granules were evenly distributed in each subplot by using a template made with a piece of woven-wire fencing material. Granules were handled individually with a forceps and delivered directly to the soil surface through a small glass funnel to avoid direct contact with the grass. Treatments at each concentration of [^{14}C]AVM were replicated 2 times.

Plants. A study was made of the absorption and degradation of [^{3}H]AVM after foliar application to cotton in the field. Mature, fully expanded cotton leaves of uniform size and age were each treated in situ with 100 μg of [^{3}H]AVM (in 150 μL of the aqueous ethanol solution) by uniformly spreading the solution with a micropipet over the upper leaf surface. Treated leaves were identified with paper tags.

A separate test was conducted to determine if [^{3}H]AVM or radioactive products of its decomposition in soil would be taken up by cotton plants grown in treated soil. For this, the aforementioned 500-g sample of soil that had been treated with [^{3}H]AVM at a rate of 10 ppm was removed from the holding container at 3 months posttreatment and mixed thoroughly. A 250-g subsample was then extracted with solvent as described (vide infra) and air-dried. Also at that time, a previously untreated 200-g sample of the same type of soil was treated with [^{3}H]AVM (10 ppm) in the same way. Thus, three different samples of soil treated with [^{3}H]AVM at 10 ppm were available for use in growing the plants: extracted and unextracted aged (3-month) samples and a freshly treated, unextracted sample. Before initiation of tests of the uptake of AVM by plants, portions of these soil samples were subjected to combustion analysis (vide infra) to determine levels of radioactive material.

Portions (200 g) of each of the samples of treated soil, as well as some control samples of untreated soil, were mixed individually with 25 g of vermiculite and placed in shallow glass containers. Water was added to thoroughly wet the soil, then eight acid-delinted cotton seeds were planted in each container. The samples were held in a greenhouse where they were watered as needed until seedlings developed to the stage of having two unexpanded true leaves.

Sample Preparation. *Soil.* Triplicate samples of the glass vials containing 10 g of treated soil were collected at the specified times posttreatment and the soil was trans-

ferred quantitatively with acetonitrile (ca. 25 mL) to a 250-mL glass-stoppered flask. The flask contents were agitated vigorously for 30 min with a wrist-action shaker, and after the solids settled, the solvent extract was decanted and the sample was extracted 2 more times with 25-mL portions of acetonitrile.

After the last extraction, samples were centrifuged to facilitate separation of soil and solvent, and the combined solvent extracts from individual samples were held for further analysis. Extracted soil was air-dried and held for combustion analysis to quantitate unextracted radioactive material. The 10-g samples treated at the 50-ppm level, and the bulk sample treated at 10 ppm, were extracted the same way with acetonitrile and then 1 additional time with a 9:1 mixture of acetone and water. (The additional extraction with aqueous acetone allowed recovery of ca. 3–5% more of the applied radioactive material.) With some of the treatments, the water in the sample holding chamber was recovered, adjusted to a specific volume, and radioassayed to obtain an estimate of ^{3}H that was released during the degradation of [^{3}H]AVM in soil and then condensed as ^{3}H-labeled water. This water was also extracted with methylene chloride (5:1 v/v) to determine if there was any accumulated radioactivity that could be attributed to volatilized organosoluble products.

Cotton Leaves. Triplicate samples of treated cotton leaves were collected at the specified times and rinsed thoroughly with methanol (ca. 100 mL) to recover unabsorbed radioactive material. Each rinsed leaf was then cut into small pieces and homogenized with a 9:1 mixture of acetone and water. Solids were separated by centrifugation and reextracted 2 times with acetone. The leaf rinses, and combined extracts of leaves, were held separately for further analysis. Extracted leaf tissues were air-dried and held for combustion analysis to determine the levels of unextracted radioactive material.

Plant Uptake. Cotton seedlings that were grown in the soil treated with [^{3}H]AVM (10 ppm) were collected, washed thoroughly with tap water, and blotted dry with paper towels. Roots were separated from the plants at the soil line, and the samples of stems plus leaves, and roots, were weighed and allowed to air-dry. Dried samples were weighed and ground in a Wiley mill, and then subsamples were analyzed for ^{3}H content by oxygen combustion (vide infra).

Bait Formulation. In the tests with bait formulations of [^{14}C]AVM, all the grass in each subplot at each sample time was cut back to a height of ca. 8 cm. The harvested grass collected from each subplot was weighed and then a subsample was stored fresh at −30 °C for possible later examination and analysis. The major portion of each sample was dried 48 h at 50 °C, reweighed, and milled, and then duplicate 250-mg subsamples from each plot and collection time were analyzed by oxygen combustion for determination of radiocarbon content. At the conclusion of these tests, two core samples of soil (2.5 × 25 cm) were taken from each plot and each was separated into three subsections (0–7.5, 7.5–15, and 15–25 cm). These samples were dried 24 h at 50 °C, pulverized with a mortar and pestle, sieved to pass a 35-mesh screen, and then held for determination of possible radiocarbon residues by combustion analysis (vide infra).

Sample Analysis. Solvent extracts and rinses were radioassayed and then evaporated under vacuum and gentle heat until just dry. Ethanol was added to samples containing water to effect azeotropic distillation. The residue was taken up in ca. 0.5 mL of acetonitrile and analyzed by thin-layer chromatography (TLC). Precoated

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glass plates (silica gel 60 F-254, 0.25 mm thick; E. Merck, Cincinnati, OH) were used with solvent mixtures (A) 10:3:1 ethyl acetate, benzene, and 2-propanol or (B) 6:4 hexane and 2-propanol. Samples were routinely developed twice in one dimension with the A mixture; selected samples were developed in two dimensions with the two different solvent mixtures. Radioactive materials were located on the TLC plates with a conventional autoradiographic procedure (^{14}C) or autofluorography (^{3}H). For autofluorography, plates were sprayed with a 10% solution of PPO (2,5-diphenyloxazole) in toluene until thoroughly wet (but not to the point of runoff). After air-drying, the sprayed plates were exposed to X-ray film (Kodak X-OMAT AR, Eastman Kodak Co., Rochester, NY) and held at -30 °C for a suitable period of exposure. Radioactive areas were scraped into scintillation vials for quantitation by liquid scintillation counting (LSC). With TLC analyses of ^{3}H-labeled materials, these areas were marked with a template so that the same amount of gel was removed with each sample; preliminary tests indicated that increasing gel content in the counting vials were accompanied by a substantial reduction in counting efficiency by LSC.

Samples (1 g) of dried extracted soil that had been treated with [^{3}H]AVM were combusted in a tube furnace at 1000 °C for 30 min in an oxygen atmosphere (flow rate 100 mL/min). The tritium released by combustion was trapped as tritiated water in a cold U-tube. Tubing connecting the furnace with the U-tube was wrapped with heating tape to prevent premature condensation of the tritiated water. At the end of each combustion period, the contents of the U-tube were transferred quantitatively to scintillation vials with LSC fluid appropriate for aqueous samples. (Trial runs with [^{3}H]AVM-spiked soil samples indicated that this combustion procedure provided >90% recovery of the tritium present). The combustion procedure used for dried plant materials treated with [^{3}H]AVM was similar to that described for soil except that the temperature of the furnace was 500 °C and each sample was held in the combustion train for 1 h. Under these conditions, combustion efficiency was ca. 85%.

Radiocarbon in plants treated with [^{14}C]AVM was determined by combusting samples in an oxygen atmosphere with a sample oxidizer (Packard Instruments Co., Inc., Downers Grove, IL) and trapping combustion gases in a mixture (1:1 v/v) of Carbosorb II (Packard Instruments, Co., Inc.) and LSC fluid. This trapping solution was subsequently mixed with an additional 10 mL of LSC fluid for radioassay. Radiocarbon residues in soil samples (1 g) were determined with a procedure similar to that used for soils treated with [^{3}H]AVM, except that combustion gases were passed through a gas dispersion tube into 20 mL of a ^{14}CO$_2$ trapping solution (1:3 v/v, Carbosorb II and LSC fluid) that was then directly radioassayed.

High-performance liquid chromatography (HPLC) was used for analyses of certain extracts of samples of soil treated with [^{3}H]AVM to determine if there was any exchange of the tritium label. Soil extracts were filtered through Millipore filters (pore size 0.5 μm) prior to injection, and sequential samples of the HPLC eluate from each analysis were collected and radioassayed to relate radioactivity content with HPLC peak area. The HPLC analyses were conducted with an instrument (Waters Associates, Inc., Milford, MA) was operated with a C$_{18}$ reversed-phase column using a solvent mixture of acetonitrile, methanol, and water (60:20:20 v/v) at a flow rate of 1 mL/min. An ultraviolet (254 nm) detection cell was used at a sensitivity setting of 0.02 AUFS. Under these conditions, the observed retention times of AVM and its monosaccharide and 5-ketone derivatives were 6.0, 5.0, and 6.78 min, respectively.

Mass spectra (MS) were obtained by using an LKB 9000 instrument with the following operating conditions: 70-eV ionization potential, 50-μA filament current, 3.5-kV accelerating potential, and 250 °C source temperature. Trimethylsilylation of samples was achieved by reaction with a mixture of bis(trimethylsilyl)trifluoroacetamide and dimethylformamide (1:1 v/v) for 30 min at room temperature. Nuclear magnetic resonance (NMR) analyses of samples in deuteriochloroform were conducted with a Varian 300-MHz superconducting spectrometer equipped with a Fourier-transform accessory. Data were collected with a flip angle of approximately 80° and an acquisition time of 1 s. Infrared (IR) spectral data of chloroform solutions of samples were obtained with a Nicolet 7199 FTIR spectrometer with a globar source, KBr optics, and a MCT detector.

RESULTS AND DISCUSSION

Laboratory Studies: Evaluation of Possible Tritium Exchange. HPLC and LSC analyses of certain extracts of soil treated with [^{3}H]AVM for use in studies to determine the potential for tritium exchange indicated that as many as three HPLC peaks visualized by UV absorption contained measurable amounts of radioactivity, and essentially all the radioactive material injected onto the column was recovered. With the procedure used, there was no evidence of the 5-ketone derivative of AVM, a potential nonlabeled product that might be formed by oxidation at the 5-position.

TLC analyses indicated that there was no apparent degradation of the [^{3}H]AVM in the "0-day" sample, but at 28-days posttreatment ca. 40% of the dose was converted to at least six other radioactive products identical with those described below from similar tests. [^{3}H]AVM in the 0 and 28 day samples was then purified with a preliminary preparative TLC step followed by isolation on HPLC. The AVM fraction thus recovered from HPLC eluates was free of UV-absorbing impurities arising from soil extraction.

Comparative quantitative analyses with HPLC and LSC indicated the specific activity of [^{3}H]AVM after 28 days exposure to soil was unchanged. That is, the ratio of micrograms of AVM determined by radioassay of the HPLC eluate containing that compound to the micrograms measured by comparing the HPLC peak with a standard was 106.3 ± 3.6 at 0 days posttreatment and 104.4 ± 3.0 at 28 days. Thus, we concluded that exchange of the ^{3}H label would not be a major problem to contend with in our soil studies. A similar conclusion has been reached by others with respect to the use of [^{3}H]AVM in plant studies (Gruber and Jacob, 1983).

Laboratory Studies. *Degradation in Soil.* The results of studies of the fate of AVM in 10-g samples of different soils are shown in Tables I–IV. At treatment rates of 0.1, 1, and 50 ppm in Lufkin fine sandy loam, the apparent half-life of [^{3}H]AVM (TLC compound 11) was between 14 and 28 days at the two lower treatment levels and 28–56 days at the 50-ppm rate (Tables I and II). At all treatment levels, the parent material was 90% degraded by 168 days posttreatment.

As many as 13 radioactive products of the degradation of [^{3}H]AVM were resolved by TLC of extracts of treated loam. (Minor products, which individually never exceeded 2–3% of the dose at any time posttreatment, are combined in tables for convenience.) TLC compound 7 was the major degradation product at all treatment levels. This metabolite was eventually isolated from combined extracts

Table I. Degradation of [³H]AVM in Lufkin Fine Sandy Loam Treated at a Rate of 50 ppm

radioactive compounds[a] (TLC R_f values)	% of applied radioactivity at indicated days posttreatment					
	0	14	28	56	112	168
1-3 (0.0-0.1)	0	0	0	0	3.1	1.7
4 (0.16)	0	1.0	1.9	4.9	6.4	5.3
5-6 (0.32-0.38)	0.6	2.4	3.6	6.1	5.0	3.2
7 (0.50)	0.4	8.1	13.1	15.3	15.5	5.9
8-10 (0.54-0.64)	0	1.5	2.1	4.0	3.3	7.0
11 (0.68)	96.0	79.0	62.9	35.0	16.8	5.8
12 (0.73)	0	0.8	0.8	8.6	9.2	5.3
13-14 (0.77-0.83)	0	0	0	1.3	2.1	1.6
unextractable	3.0	2.4	3.0	6.2	8.5	12.2
trapped volatiles	0	0.3	1.9	7.8	16.6	27.6
lost	0	4.5	10.7	10.8	13.5	24.4

[a] Compound 7 is AVM-OH/ALD and 11 is AVM (see Figure 1 for structures; trapped volatiles represent radio-assays of water in holding chamber.

of loam treated with [³H]AVM at the 50-ppm level and purified via preparative TLC and HPLC. On the basis of the results of MS and NMR analyses, we have concluded that metabolite 7 is an equilibrium mixture of the 8α-hydroxy derivative and the corresponding ring-opened aldehyde derivative of AVM (Figure 1) present in an approximate 1:2.5 ratio. Both the MS fragmentation patterns and the NMR features of metabolite 7 suggested that the structural transformation involved the lower portion of the macrocyclic ring. Collectively, the MS data indicate the presence of a new oxygen function that can be tri-
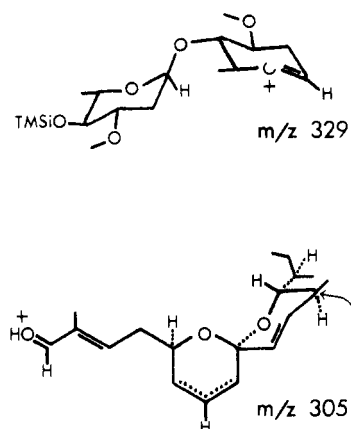

Figure 2. Key diagnostic ions from mass spectral analyses of major degradation product (metabolite 7) of AVM in soil.

methylsilylated: (1) An ion of m/z 600 in the spectrum of the unknown likely arises from the aglycon (formed in the mass spectrometer) of monooxyygenated AVM (molecular weight of this aglycon would be 600). In contrast, the aglycon of AVM has a molecular weight of 584, 16 mass units lower. (2) That the oxygenation has occurred on the macrocyclic part of the AVM molecule and not on the sugar moiety is confirmed by the observation that the ion of m/z 329 (Figure 2, top) previously reported in the spectrum of trimethylsilylated AVM (Miwa et al., 1982) is also found in the spectrum of derivatized metabolite 7. (3) Further, the trimethylsilyl derivative of metabolite 7

Table II. Degradation of [³H]AVM in Lufkin Fine Sandy Loam Treated at Rates of 0.1 and 1.0 ppm

days posttreatment	distribution of radioactivity, % of dose in							
	compounds[a]						unextr	lost
	4	5-6	7	11	12	13-14		
	0.1 ppm							
0	0	0	0	95.1	0	0	4.9	0
7	0	0	0	93.2	0	0	4.9	1.9
14	0	2.6	7.3	67.3	5.3	0	6.8	10.7
28	4.8	3.6	16.7	44.4	3.3	0	15.5	11.7
56	6.9	4.0	18.5	21.6	9.2	0	21.4	18.4
84	7.9	3.6	17.0	15.4	5.0	0.6	30.1	20.4
168	7.4	0	13.3	5.3	4.1	0	35.0	34.9
	1.0 ppm							
0	0	0	0	94.7	0	0	5.3	0
7	0	0	5.1	83.1	0	0	6.0	5.8
14	0	0	12.3	60.9	8.6	0	7.3	10.9
28	6.3	0	17.4	35.5	11.8	0	9.3	19.7
56	10.4	3.1	20.1	18.0	11.9	0.8	17.6	18.1
84	8.6	2.6	14.8	9.1	4.7	0.5	23.7	36.0
112	6.7	2.5	13.5	7.1	3.2	0.5	27.5	39.0
168	7.9	8.8	5.0	3.6	2.9	0	19.8	52.0

[a] See Table I for TLC R_f values.

Table III. Degradation of [³H]AVM in Sand Treated at a Rate of 1.0 ppm

days post-treatment	distribution of radioactivity, % of dose in									
	compounds[a]							unextr	vol	lost
	4	5-6	7	8-10	11	12	13-14			
0	0	0	0	0	99.2	0	0	0.8	0	0.0
14	0.6	1.4	6.4	6.2	65.8	10.6	1.3	2.5	0.7	4.5
28	1.0	3.1	9.7	3.5	64.9	6.5	0.7	3.8	2.9	3.9
56	1.7	2.3	13.2	2.8	47.4	9.0	1.9	7.2	8.2	6.3
84	3.1	2.7	18.2	0	40.1	8.0	2.5	7.1	11.7	6.6
112	3.2	2.4	15.1	2.8	22.9	7.8	3.0	11.8	16.5	14.5
168	5.5	0	20.1	0	21.9	7.6	4.2	12.5	22.5	5.7
252	6.4	2.8	15.8	0	9.8	6.1	3.9	17.3	28.0	9.9

[a] See Table I for TLC R_f values.

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Table IV.   Degradation of [³H]AVM in Houston Clay Treated at Rates of 0.1 and 1.0 ppm

| days post-treatment | distribution of radioactivity, % of dose in | | | | | | | | | |
| | compounds[a] | | | | | | | unextr | vol | lost |
	4	5-6	7	8-10	11	12	13-14			
					0.1 ppm					
0	0	0	0	0	94.9	0	0	5.1		0
21	3.0	4.2	11.2	0	54.6	8.8	0	9.1		9.1
28	5.0	4.7	13.4	0	47.8	10.9	0	13.1		5.1
56	9.7	0	18.4	0	29.6	12.1	0	17.2		13.0
84	11.6	1.1	18.7	0	19.4	9.8	0	20.2		19.2
112	10.3	0	14.4	0	12.5	8.3	0	21.2		33.3
168	9.9	0	14.3	0	12.0	7.0	1.2	26.3		29.3
252	7.7	2.5	13.7	0	7.5	5.0	0	21.2		42.4
					1.0 ppm					
0	0	0	0	0	94.4	0	0	5.6	0	0
28	1.4	1.7	4.9	0	60.4	8.4	0	10.1	2.6	10.5
56	2.6	2.3	6.0	2.1	51.6	7.3	2.3	11.5	6.6	7.7
84	7.5	2.8	13.0	2.5	22.4	8.4	0	17.0	12.6	13.8
112	8.1	0	14.8	0	22.7	11.8	2.9	15.8	17.9	6.0
168	7.7	2.1	8.5	6.2	11.3	7.2	1.4	18.8	25.6	11.2
252	4.2	2.5	11.4	0	11.2	7.4	1.4	18.1	33.4	10.4
448	2.1	4.2	5.2	5.1	8.1	4.3	0	16.8	45.5	8.7

[a] See Table I for TLC R_f values.

yields an ion of m/z 726, as does the trimethylsilyl derivative of the known in vitro steer liver metabolite of AVM in which hydroxylation has occurred on the C-24 methyl (Miwa et al., 1982). The analogous ion from derivatized AVM is found at m/z 638, reflecting a mass difference of 16 + 72 (an additional oxygen atom that undergoes trimethylsilylation). (4) The ion of m/z 305 (Figure 2, bottom) found in the spectra of the parent compound and its trimethylsilyl derivative is also present in the spectra of metabolite 7 and its trimethylsilyl derivative. This is in contrast to the MS data obtained from the in vitro liver metabolite described by Miwa et al. (1982) because, with the C-24 hydroxymethyl compound, ions of m/z 321 (free) and 393 (Me₄Si), but not 305, were reported. Thus, the introduction of an oxygen atom to form the soil degradation product must have occurred in the "lower portion" of the AVM molecule and not on the sugar moiety or on that part of AVM that yields the m/z 305 ion.

The need to incorporate a hydroxyl group initially posed problems in the interpretation of the NMR spectra. While the absence of a characteristic 8α-methylene signal pointed to C₈α as the probable hydroxylation site, the inability to detect a hemiacetal proton peak near δ 5 raised doubts about this structure. It was also difficult to understand why the chemical shifts of the 24-methyl and a methoxyl, protons remote from C₈α, were displaced from their positions in the parent molecule. These problems were resolved once it was sensed that the signal at δ 10.0, most reasonably assigned to a formyl proton, could arise from the ring-opened form of the hemiacetal. Since the isolate was a 2.5 to 1 mixture of the AVM-related components, it was reasonable to conclude that the spectrum represented an equilibrium mixture of the aldehyde and hemiacetal with the aldehyde predominating. This view accounted for the apparent nondetectability of the hemiacetal proton since its intensity would be only about 30% of stoichiometry. It also provided an explanation for the distant chemical shift displacements since opening of the fused ring system could well affect the stereochemical relationships in the entire molecule. The IR spectrum of the AVM soil degradation product showed three distinctive peaks at 1625, 1657, and 2857 cm⁻¹ that are not present in the spectrum of AVM. The positions and relative intensities of these bands indicate the presence of an α,β,-γ,δ-unsaturated aldehyde, which is in full agreement with the proposed structure.

Substantial quantities (ca. 30% of dose through 168 days posttreatment) of radioactive material condensed in the water used to maintain high levels of relative humidity (RH) in containers holding samples of loam treated with 50 ppm of [³H]AVM (Table I). Since none of this radioactive material partitioned into methylene chloride, we have concluded that it more than likely represents tritiated water rather than volatile organic materials. Because special tests (vide supra) indicated there was no apparent tritium exchange upon aging of [³H]AVM in treated soil, we speculate that the apparent release of tritium resulted from metabolic oxidation at the C-5 position of the parent molecule or a degradate.

Two-dimensional TLC analyses indicated that TLC compound 13 cochromatographed with the monosaccharide derivative of AVM. Other unidentified degradation products that accounted for 5% or more of the applied [³H]AVM in treated loam included TLC compound 12, which was slightly less polar than the parent compound in the TLC systems used and a polar metabolite (TLC compound 4).

Similar studies with sand treated with [³H]AVM at a rate of 1.0 ppm (Table III) and with clay treated at rates of 0.1 and 1.0 ppm (Table IV) indicated that the array of degradation products detected was the same as found in loam; again, metabolite 7 predominated but metabolites 4 and 12 were also present in significant quantities. The half-life of [³H]AVM in sand was between 28 and 56 days, while in clay the half-life was ca. 28 days at the 0.1-ppm treatment level and 56 days at 1.0 ppm. In these soils, >90% degradation of AVM had occurred by ca. 252 days posttreatment. Significant levels (ca. 30%) of radioactive material also condensed in the water-filled reservoirs of containers used to hold samples of sand or clay treated at the 1.0-ppm level with [³H]AVM.

In all three soils treated with [³H]AVM, the concentration of bound, unextractable radioactive material tended to increase progressively with time posttreatment. With the exception of sand, there was also a progressive increase in the fraction (designated "lost") of the applied dose of [³H]AVM that could not be accounted for in a radiobalance assessment. That this loss also was observed among samples held in containers where condensed volatile radioactive material was measured suggests that the trapping

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Table V. Radioactive Material in Cotton Seedlings
Grown in Lufkin Fine Sandy Loam Treated with
[³H]AVM (10 ppm)

| | ppm of [³H]AVM equiv | | |
| | dry weight, in soil | wet weight | |
soil sample		in stem/ leaf	in root
unaged, unextracted	10.0	0.08	3.60
aged 3 months, unextracted	6.4	0.12	3.26
aged 3 months, extracted	1.9	0.02	0.15

of these volatiles was inefficient and that the concentrations of such material(s) may have been higher than what we report.

Results of studies of the fate of [¹⁴C]AVM following treatment of Lufkin fine sandy loam at a rate of 1 ppm were similar to those obtained in studies with [³H]AVM. The array of the major radioactive degradation products and the rates of their formation from the two radiolabeled preparations were essentially the same in samples held under aerobic conditions. During a 3-month test period, there was no apparent degradation of [¹⁴C]AVM in samples where anaerobic conditions were established immediately after treatment; at all time posttreatment, solvent extraction of treated soil resulted in quantitative recovery of the applied dose as unmetabolized AVM. The observed lack of unextractable radioactive material in these samples held under completely anaerobic conditions suggests that the progressive and extensive accumulation of such residues in samples held aerobically is attributable to binding between soil and AVM degradation products but not to the parent compound. In the samples held 1 month under aerobic conditions before change to anaerobic conditions during the ensuing 3 months, there was definite reduction in the rate at which the parent compound was degraded. For example, in samples extracted and analyzed at 3 months after establishment of anaerobic conditions, ca. 42% of the applied dose was still in the form of AVM compared with ca. 18% in samples treated at the same time but held continuously under aerobic conditions.

Leaching. [³H]AVM showed no leaching potential in any of the soils tested; the compound remained at the origin of all soil/TLC plates after development in water. This result was anticipated because this compound is virtually insoluble in water (ca. 8 ppb). The two compounds used as analytical standards in these tests had, as expected, the same leaching characteristics reported by Bull and Shaver (1980); that is, diflubenzuron remained at the origin of all soil/TLC plates while PDIC leached readily in each case.

Biometer Flasks. Weekly analyses of the alkaline trapping solutions used in side arms of biometer flasks containing Lufkin fine sandy loam treated with [¹⁴C]AVM (10 ppm) indicated that consistent but very low levels of radioactive material were being trapped during each sample interval. Through 21 weeks posttreatment, the accumulated amount trapped was only 3.2% of the applied dose. Verification studies indicated that all of this trapped radioactive material was $^{14}CO_2$. Evolution of $^{14}CO_2$ indicates there was some as yet unidentified structural fragmentation of [¹⁴C]AVM in soil; however, the low levels of $^{14}CO_2$ trapped do not suggest extensive oxidation of the carbon–carbon bond at the labeled positions accompanied by the elimination of CO_2 as a major pathway in soil under the conditions of these tests.

Uptake by Cotton Seedlings. The results of tests to determine if [³H]AVM or radioactive products of its deg-

radation would be taken up by cotton seedlings grown in treated soil are shown in Table V. Low levels of radioactive material were detected in all the plants exposed to the three different treatments. The least amounts of radioactive material were recovered from plants grown in soil previously extracted with solvents at 3 months posttreatment, while greater (and similar) amounts were found in plants grown either in unextracted unaged treated soil or in unextracted soil aged for 3 months posttreatment. Very little radioactive material (ca. 0.1 ppm) was found in stems and leaves of seedlings from the latter two treatments but >3 ppm was associated with roots. Whether the radioactive material in roots was absorbed or simply adsorbed on the external surface of the tissues is not known. It is apparent, however, that neither [³H]AVM nor its radioactive products were taken into the vascular system of seedlings in major quantities.

Field Tests. *Cotton.* Studies of the fate of [³H]AVM after application to cotton leaves demonstrated that the compound was rapidly degraded on leaf surfaces (Table VI). Although radiobalance data indicated slightly more than half the applied radioactivity remained on leaves at 2 days posttreatment, only one-third of the recovered radioactive material was AVM. We presume that this apparent instability of AVM on foliar surfaces resulted from photodegradation. TLC analyses of the external rinses of treated leaves indicated that radioactive material on the plates moved with heavy streaking from the origin to the solvent front with poor resolution of individual products other than the parent compound. Little radioactive material was recovered from the internal extracts of treated leaves; a maximum of 8.0% of the applied radioactive material was measured in leaves at 8 days posttreatment, and only 1.4% of that was AVM. In addition, little radioactive material (maximum of 6.5% at 8 days) remained in unextractable form in leaf tissues. Substantial portions of the applied dose (ca. 73% after 8 days) could not be accounted for, and these losses of radioactive material increased progressively over time. Since AVM is nonvolatile, and there was no rainfall during these tests, such losses may be attributable to a volatization of radiolabeled fragments following photodecomposition of the molecule.

Fire Ant Bait Formulation. The results of combustion analyses of Bermuda grass harvested after treatment of plots with a granular formulation of [¹⁴C]AVM at rates equivalent to 50, 150, and 500 mg of AI/acre indicated that very little if any radioactivity was taken up into in any of the samples. None of the samples analyzed contained more than a few cpm above background, and it is possible that this could be explained on the basis of random variation in counting rather than by the presence of radiocarbon (specific activity of [¹⁴C]AVM in the counting system used was 30 000 cpm/μg). Concurrent combustion analyses of standards consisting of 250-mg samples of untreated grass fortified with known amounts of [¹⁴C]AVM indicated that the combustion procedure used gave >95% recovery of the radiocarbon present.

A 100-g sample of fresh grass collected at 2 weeks posttreatment from the plot treated with the [¹⁴C]AVM bait formulation at the 500 mg of AI/acre rate was homogenized with acetone (1:20 w/v). The solvent extract was filtered and evaporated, and then subsamples (100 mg/ each) of the viscous residue (2 g) that remained were analyzed via combustion. Radioassay of these samples indicated there were no more than 300 cpm (or ca. 0.01 μg)/g of extracted concentrated residue. On the basis of these data, equivalent residues in fresh grass would not exceed 0.2 ppb.

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Table VI. Fate of [³H]AVM after Foliar Application to Cotton Leaves in the Field (ca. 100 μg/Leaf)

days post-treatment	distribution of radioactivity, % of dose (±SE)[a]					
	external rinse		internal rinse			
	total	AVM	total	AVM	unextr residue	lost
0	97.6 ± 1.2	97.6	2.3 ± 0.6	2.3	0.1	0.0
1	74.0 ± 1.3	27.3	3.2 ± 0.3	2.5	0.5	22.3
2	53.9 ± 1.8	18.4	5.3 ± 0.8	3.2	3.2	37.6
4	25.6 ± 1.0	5.1	7.0 ± 0.4	2.5	6.2	61.2
8	12.9 ± 1.5	1.8	8.0 ± 0.7	1.4	6.5	72.6

[a] Data represent averages of six or more replicates per time. Figures represent percent of dose accounted for by [³H]-AVM as determined by TLC of pooled samples; balance of each sample is uncharacterized radioactive material.

Table VII. Distribution of Radioactive Material after Application of Fire Ant Bait Formulation of [¹⁴C]AVM to Soil

days posttreatment	μg of [¹⁴C]AVM equiv in			
	granules		soil	
	extract	residue	extract	residue
0	2.97	<0.01		
3	1.14	0.02	1.29	0.56
7	0.84	0.12	0.79	0.96

An attempt was made to determine more directly the fate of [¹⁴C]AVM in granules after their application to soil. For this, AVM granules were placed on the surface of Lufkin fine sandy loam (3 g) contained in perforated stainless steel planchets (25 mm diameter × 7 mm deep). These samples (10 granules/planchet) were positioned in field soil so that the top of the planchet was approximately flush with the surface of the soil. Samples were collected at the specified times and extracted thoroughly with acetone. Extracts were radioassayed, and samples of extracted granules and soil were subjected to combustion analyses. The results (Table VII) indicated that ca. 62 and 59% of the dose of [¹⁴C]AVM was recovered from the soil surrounding the granules by 3 and 7 days posttreatment, respectively. Of the [¹⁴C]AVM that was associated with the soil, ca. 70 and 40% was recovered by solvent extraction at 3 and 7 days posttreatment, respectively. TLC analyses indicated that at 7 days posttreatment only ca. 16 and 24%, respectively, of the radioactive material extracted from soil and granules represented [¹⁴C]AVM.

Casual observations of these samples indicated that some of the oil from the granules had apparently soaked into the soil. Since AVM is oil soluble, movement of oil into adjacent soil may well account for the loss of AVM from the granules. (This test was terminated after 7 days because a rainstorm destroyed the samples and there were no granules available for further testing.)

Combustion analyses of duplicate 1-g subsamples of soil cores collected 8 weeks after treatment of soil with granular formulations of [¹⁴C]AVM indicated there was very little if any radioactive material in any of the samples from core segments of different depths (i.e., a range of no more than 1-15 cpm/g above background in samples from 0 to 7.5-cm depth, 0 cpm at 7.5-15 cm, and 0-4 cpm at 15-25 cm). Similarly, analyses of coarse surface materials that did not pass through the sieve indicated no more than a total of 50 cpm above background in any sample. Clearly, the potential radioactivity observed was well below the level of reliable measurement and may be the result of random variation. The observed low level of radiocarbon in surface materials was surprising only because these samples possibly included some of the original granules picked up during sampling. However, it should be noted that rainfall was high during the experimental period (16 days of

measurable rainfall, total of 26 cm), and this in conjunction with degradation in granules and soil may have had a major influence on the apparent rapid dissipation of AVM from the treated area.

CONCLUSIONS

These studies have shown that, under laboratory conditions, AVM degrades progressively at fairly rapid rates in representative agricultural soils such as Lufkin fine sandy loam and Houston clay, as well as in sand. It seems likely that rates of AVM degradation in the field would be accelerated over those seen under laboratory conditions. Considering that AVM appears to have little leaching potential, it seems likely that inadvertent contamination of soil surfaces with AVM during field application will not lead to serious problems with persistent residues or with contamination of surface or subterranean water. Results of studies of the uptake of residues of AVM from soil by cotton seedlings suggest that, even if residues of AVM or its degradation products are present in soils, their uptake by plants grown in contaminated soil most likely would be very minor. The studies with a fire ant bait formulation of AVM indicate that under conditions of moderate to heavy rainfall there is little chance for appreciable contamination of soil or of grass grown in the soil treated with the granules at the anticipated (50 mg of AI/acre) or higher (100-500 mg/acre) use rates.

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Degradation of the Insecticide Azinphos-methyl in Soil and by Isolated Soil Bacteria

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The insecticide azinphos-methyl (I) is degraded in soil to a great extent after a 197-day incubation period. Liberation of two benzoic acid derivatives (benzamide, XV, and salicylic acid, XVI) and of $^{14}CO_2$ from [*carbonyl*-^{14}C]- and [*ring*-U-^{14}C]azinphos-methyl indicates that the 1,2,3-benzotriazinone ring is cleaved in soil. Of the 17 metabolites identified, 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3H)-one (VI), 3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3H)-one (XI), and the corresponding sulfinyl (V) and sulfonyl (IV) derivatives represent key intermediates in azinphos-methyl degradation in soil. Compounds XI, V, and IV are cleaved by *Pseudomonas fluorescens* DSM 1976, forming anthranilic acid as the main metabolite, thus demonstrating that the insecticide can be metabolized in soil via easily decomposable benzoic acid derivatives.

Although there is some information available concerning the degradation of the insecticide azinphos-methyl [*O,O*-dimethyl *S*-[[4-oxo-1,2,3-benzotriazin-3(4H)-yl]methyl] phosphorodithioate] both in soil and by isolated soil bacteria, the fate and exact degradation pathway of this compound in soil systems still remain unclear. This fact is mainly due to the polar character of the metabolites, thus forming nonextractable residues, as well as to problems arising during structure elucidation of such degradation products that often show significant thermal lability.

The main metabolites already described during degradation studies of azinphos-methyl in various soils are benzazimide, (thiomethyl)benzazimide, *N*-methylbenzazimide sulfide, and bis(benzazimidyl-methyl) disulfide. They were identified, however, only by chromatographic methods (Schulz et al., 1970). More recently we demonstrated that the soil bacterium *Pseudomonas fluorescens* DSM 1976 degrades azinphos-methyl to bis(benzazimidylmethyl)disulfide [bis[3-(thiomethyl)-1,2,3-benzotriazin-4(3H)-one] and benzazimide [1,2,3-benzotriazin-4-(3H)-one] via hydrolysis and enzymatic oxidation as well as to anthranilic acid by cleavage of the heterocyclic ring (Engelhardt et al., 1981). A great number of other soil bacteria of the genera *Pseudomonas* and *Arthrobacter* also proved capable of cleaving the 1,2,3-triazinone ring of this insecticide, forming anthranilic acid (Wallnöfer et al., 1982). Since anthranilic acid was not formed via benzazimide (or benzazimidylmethanethiol) as an intermediate, the reactions leading to anthranilic acid remained to be elucidated.

In the present study the metabolites formed during degradation of [*carbonyl*-^{14}C]- and [*ring*-U-^{14}C]azinphos-methyl in different soil types were isolated and identified by means of nuclear magnetic resonance and mass spectroscopy and determined quantitatively by measuring the distribution of ^{14}C activity. In addition, some of the major transformation products of azinphos-methyl in soil, which represent structures related to the starting compound, were subjected to further degradation by *P. fluorescens* DSM 1976 to obtain more information on the biodegradability of these substances and on the pathway by which anthranilic acid is formed from the insecticide in this bacterium.

EXPERIMENTAL SECTION

Chemicals. [*carbonyl*-^{14}C]Azinphos-methyl (specific activity 2.7 mCi/mM) and [*ring*-U-^{14}C]azinphos-methyl (specific activity 25 mCi/mM) were synthesized by Mobay Chemical Corp., Agricultural Division, Kansas City, MO. The synthetic reference compounds of intermediates were prepared by Bayer AG, Leverkusen, West Germany, Abteilung Pflanzenschutz-Chemische Forschung II (Dr. Cölln) and Abteilung Pflanzenschutz-Anwendungstechnik (Dr. Wagner).

Organisms and Culture Conditions. *P. fluorescens* DSM 1976 was cultured in 100 mL of Hegeman's mineral base (Hegeman, 1966) with additional 0.02% yeast extract, 160 mg of the respective substrate/L and 4 g/L disodium fumarate as the carbon source as described (Engelhardt et al., 1981).

Soil Types Used. Soil degradation studies were performed according to the BBA (1980) (Biologische Bundesanstalt Braunschweig, West Germany). The two soil types used were native soil from Laacherhof Experimental Station (organic matter 0.8%; clay 19.8%; silt <20 μm 34.9%; moisture content 11.2%; pH 6.0) and standard soil no. 1 (organic matter 2.66%; silt 14.9%; particle sizes <0.002 mm 5.8%, 0.002–0.02 mm 9.1%, 0.02–0.2 mm 41.8%, and >0.2 mm 43.3%; pH 6.1). Prior to insecticide addition, the standard soil sample (about 2 kg) was adjusted to 40% maximum water capacity by using distilled water (11 g of H_2O/100 g of soil) and incubated for 14 days at 22 °C in the dark. The water that evaporated was replenished at a 3-day interval.